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Signed D. Mahoney

Dated 11 August 2000

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Request for grant of a patent

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29 JUL 1999

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference
C1064.00/C

2. Patent application number
(The Patent Office will fill in this part)
9917816.2
29 JUL 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
Cytocell Limited
Unit 6 Somerville Court
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Banbury, Oxon, OX17 3SN

Patents ADP number *(if you know it)*
6048755003

If the applicant is a corporate body, give the country/state of its incorporation
United Kingdom

4. Title of the invention
Chemically Modified Probes used to Regulate Nucleic Acid Amplification Assays

5. Name of your agent *(if you have one)*
Keith W Nash & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*
90-92 Regent Street
Cambridge
CB2 1DP

Patents ADP number *(if you know it)*
1206001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
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Continuation sheets of this form	
Description	18
Claim(s)	1
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature KEITH W NASH & CO, AGENTS Date 29.07.1999

12. Name and daytime telephone number of person to contact in the United Kingdom: Mrs H C Matthews (01223) 355477

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Title: Chemically Modified Probes used to Regulate Nucleic Acid Amplification Assays.

The present invention relates to the introduction of linker/spacer molecules into oligonucleotide probes for the improvement of nucleic acid amplification processes, for use in such processes and to kits comprising such probes for performing such processes. The present invention is concerned with amplification of hybridised modified nucleic acid probes such that specificity of the reaction is increased.

Summary of the invention: Disclosed is a method of amplifying a nucleic acid sequence of interest, comprising: an oligonucleotide probe consisting of sequences complementary to the nucleic acid sequence of interest. Upon hybridisation to the region of interest, portions of said sequences anneal, at least in part, such that chain extension of at least one of the nucleic acids may be undertaken. The oligonucleotide probe is designed such that it incorporates an RNA polymerase promoter sequence and related sequences, together with a sequence of choice such that upon subsequent hybridisation and chain extension of the nucleic acid sequence of interest, a functional double stranded RNA polymerase promoter is formed. In the presence of RNA polymerase, RNA is synthesised, the sequence of which is designed such that it is complementary in part to a second oligonucleotide probe that also contains a sequence for an RNA polymerase promoter and sequences that are complementary in part to the original sequence of interest. Upon hybridisation of the *de novo* synthesised RNA to the second oligonucleotide probe, chain extension of the RNA may be undertaken. In the presence of RNA polymerase *de novo* RNA is synthesised that is complementary in part to the first oligonucleotide probe such that a cycle is formed. Both the first and second oligonucleotide probes have incorporated linker/spacer molecules that prevent single strand readthrough of RNA polymerase and also DNA polymerase side reactions that lead to background in the absence of the sequence of interest.

Background of the Invention

A number of nucleic acid amplification processes are described in the *prior art*. One such process known as polymerase chain reaction (PCR) is disclosed in US patents nos. 4683195 and 4683202. The PCR process consists of nucleic acid primers that anneal to opposite strands of a DNA duplex; these primers are extended using thermostable DNA polymerase in the presence of nucleotide triphosphates to yield two duplex copies of the original nucleic acid sequence. Successive cycles of denaturation, annealing and extension are undertaken to further amplify copies of the original nucleic acid sequence. This method has its drawbacks including the need for adjusting reaction temperatures alternately between intermediate (e.g. 50°C-55°C) and high (e.g. 90°C-95°C) temperatures involving repeated thermal cycling. Also the time scale required for multiple cycles of large temperature transitions to achieve amplification of a nucleic acid sequence and the occurrence of sequence errors in the amplified copies of the nucleic acid

sequence is a major disadvantage as errors occur during multiple copying of long sequence tracts. Additionally, detection of the amplified nucleic acid sequence generally requires further processes e.g. agarose gel electrophoresis.

Alternative nucleic acid amplification processes are disclosed in WO no. 88/10315 (Siska Diagnostics), European patents nos. 329822 (Cangene) and 373960 (Siska Diagnostics), US patent no.5554516 (Gen-Probe Inc.) and WO nos. 89/1050 & 88/10315 assigned to Burg *et al* and Gingeras *et al* respectively. These amplification processes describe a cycling reaction comprising alternate DNA and RNA synthesis. This alternate RNA/DNA synthesis is achieved principally through the annealing of oligonucleotides adjacent to a specific DNA sequence whereby these oligonucleotides comprise a transcriptional promoter. The RNA copies of the specific sequence so produced, or alternatively an input sample comprising a specific RNA sequence (US 5554516), are then copied as DNA strands using a nucleic acid primer and the RNA from the resulting DNA:RNA hybrid is either removed by denaturation (WO 88/10315) or removed with RNase H (EP 329822, EP 373960 & US 5554516). The annealing of oligonucleotides forming a transcription promoter is then repeated in order to repeat RNA production. Amplification is thus achieved principally through the use of efficient RNA polymerases to produce an excess of RNA copies over DNA templates. The RNase version of this method has great advantages over PCR in that amplification can potentially be achieved at a single temperature (i.e. isothermally). Additionally, a much greater level of amplification per cycle can be achieved than for PCR i.e. a doubling of DNA copies per cycle for PCR; 10-100 RNA copies per cycle using T7 RNA polymerase. A disadvantage associated with the DNA:RNA cycling method described in EP 329822 is that it requires test nucleic acid with discrete ends for the annealing of oligonucleotides to create the transcriptional promoter. This poses difficulties in detection of, for example, specific genes in long DNA molecules. Further disadvantages of this method are that at least three enzymes are required to undertake the DNA:RNA cycling with potentially deleterious consequences for stability, cost and reproducibility; and that one or more further processes are often required (e.g. gel electrophoresis) for detection of the amplified nucleic acid sequence.

The processes described above all refer to methods whereby a specific nucleic acid region is directly copied and these nucleic acid copies are further copied to achieve amplification. The variability between various nucleic acid sequences is such that the rates of amplification between different sequences by the same process are likely to differ thus presenting problems for example in the quantitation of the original amount of specific nucleic acid.
The processes listed above have a number of disadvantages in the amplification of their target nucleic acid; therefore, a list of desiderata for the sensitive detection of a specific target nucleic acid sequence is outlined below;

a) the process should not require copying of the target sequence.
b) the process should not involve multiple copying of long tracts of sequence.

c) the process should be generally applicable to both DNA and RNA target sequences including specific sequences without discrete ends.
d) the signal should result from the independent hybridisation of two different probes, or regions of probe, to a target sequence.
e) the process should include an option for detection of hybridised probe without any additional processes.

A nucleic acid amplification process that fulfils the above desiderata is disclosed in WO no. 93/062-40 assigned to Cytocell Ltd. Two amplification processes are described, one thermal and one isothermal. Both the thermal and isothermal versions depend on the hybridisation of two nucleic acid probes of which regions are complementary to the target nucleic acid. Portions of said probes being capable of hybridising to the sequence of interest such that the probes are adjacent or substantially adjacent to one another, so as to enable complementary arm specific sequences of the first and second probes to become annealed to each other. Following annealing, chain extension of one of the probes is achieved by using part of the other probe as a template. Amplification of the extended probe is achieved by one of two means; in the thermal cycling version thermal separation of the extended first probe is carried out to allow hybridisation of a further probe, substantially complementary to part of the newly synthesised sequence of the extended first probe. Extension of the further probe by use of an appropriate polymerase using the extended first probe as a template is achieved. Thermal separation of the extended first and further probe products can act as a template for the extension of further first probe molecules and the extended first probe can act as a template for the extension of other further probe molecules.
In the isothermal version, primer extension of the first probe creates a functional RNA polymerase promoter that in the presence of a relevant RNA polymerase transcribes multiple copies of RNA. The resulting RNA is further amplified as a result of the interaction of complementary DNA oligonucleotides containing further RNA polymerase promoter sequences, whereupon annealing of the RNA on the DNA oligonucleotide and a subsequent extension reaction leads to a further round of RNA synthesis. This cyclical process generates large yields of RNA, detection of which can be achieved by a number of means.

The present invention details the generation of large yields of RNA from a cyclical process with a high signal to noise ratio output. This is achieved through the hybridisation of RNA generated from an initial specific amplification reaction to a complementary DNA oligonucleotide probe containing a RNA polymerase promoter sequence, whereupon annealing of the RNA to the DNA oligonucleotide probe and a subsequent extension and transcription reaction leads to a further round of RNA synthesis. The RNA generated in this reaction is complementary in part to a second DNA oligonucleotide probe containing a RNA polymerase promoter sequence, whereupon annealing of the RNA to the second DNA oligonucleotide probe and a subsequent extension reaction leads to a further round of RNA synthesis. The RNA generated in this reaction is identical to the input RNA that is complementary entirely or in part to the first DNA oligonucleotide probe

and hence a cycle of annealing, extension and RNA synthesis is created. The DNA oligonucleotide probes incorporate linker molecules that prevent single strand readthrough of RNA polymerase, DNA polymerase side reactions that lead to noise in the absence of the input RNA sequence of interest and non-specific annealing and extension of RNA molecules to the DNA probes. Therefore, noise due to these non-specific reactions in the absence of RNA target is reduced.
Linker or spacer molecules have been used to introduce non-nucleotide segments into the oligonucleotide probes. These molecules have been used to form folds and hairpins to bridge sections of oligonucleotides where no appropriate binding is possible, as well as simply to create blocks for non-desirable enzymatic side reactions. A variety of such spacer molecules are available.

In the present invention spacer molecules in the DNA oligonucleotide probes effectively reduce noise by preventing single strand readthrough of RNA polymerase, DNA polymerase side reactions and reduce non-specific annealing and extension of RNA molecules. Input RNA for amplification can be derived from three way or two and a half way junctions, essentially as described in WO. no's. 93/062-40, 99/002-69 and 99/002-65 assigned to Cytocell Ltd.

In one embodiment, the oligonucleotide probes are designed such that the linker/spacer molecule/s are located 3' to the –8 or –5 region with respect to the beginning of the RNA polymerase promoter sequence.

In a preferred embodiment, the linker molecule is hexaethylene glycol (Hex) (see Figure 1) present singularly, singularly spaced, tandem or tandemly spaced, up to *n* times (where *n* can be any figure ≥1)

In a second embodiment, other linkers/spacers can be used. These include, but are not limited to, Inosine, Virazole, Nebularin, Nitropyrrole, Ribose, Propyl or combinations of the above eg. Propyl-Hex-Propyl, Propyl-Hex-Hex-Propyl, etc. Propyl may be replaced by, but not limited to Ethyl, Butyl, Octyl, Pentyl, Heptyl, etc.

In a further embodiment, the 3' portions (upstream of the –5 or –8 regions of the RNA polymerase promoter sequence) of the two oligonucleotide probes may be composed of Peptide Nucleic Acid (PNA) or Locked Nucleic Acid (LNA) and the remainder of the oligonucleotide probe be composed of DNA. The 3' portion of the oligonucleotide probe may contain a linker or combination of linker/spacer molecules (see Figure 2).

The 5' portion of the DNA oligonucleotide probes may contain termination sequences together with a linker, or combination of linker/spacer molecules acting as RNA synthesis terminators (see Figure 3).

In a further embodiment, the first oligonucleotide probe will contain complementary sequences to the input RNA in the 3' region and a unique sequence of choice such as, but not limited to, an RNA polymerase promoter

sequence followed by probe detection and capture sequences together with sequences identical to the 3' portion of the second oligonucleotide probe (see Figure 4).

In a further embodiment, the second oligonucleotide probe will contain complementary sequences to the RNA synthesised off the first oligonucleotide probe in the 3' region and a unique sequence of choice such as, but not limited to, an RNA polymerase promoter sequence followed by probe detection and capture sequences together with sequences identical to the 3' portion of the first oligonucleotide probe (see Figure 5).

The 3' terminus of the oligonucleotide probes when composed of DNA or RNA, should be blocked to prevent chain extension should snapback occur. Those skilled in the art will appreciate how this should be achieved i.e. 3'phosphate, 3' dideoxynucleotide, 3' Hexaethylene glycol, 3' propyl, etc. (see Figure 6).

In a further embodiment, upon annealing of the RNA molecules to either the first or second oligonucleotide probes, chain extension is achieved with, but not limited to, a DNA polymerase, using the oligonucleotide probe as template. Upon extension, an active RNA polymerase promoter is formed, that in the presence of RNA polymerase, rNTP's and correct buffer constituents, *de novo* RNA is formed. It will be evident to those skilled in the art as to how a cycling of RNA synthesis is achieved (see Figure 7).

Example 1

This example demonstrated the use of Hexaethylene glycol (Hex) linkers as blockers of RNA polymerase single strand read through and other non-specific side reactions caused by DNA polymerase. Hex linkers were incorporated at 0, 13, 25 and 37 bases from the 3' end of the template. In addition, Hex linkers were incorporated at all three positions.

Preparation of oligonucleotides

All oligonucleotide probes were synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser according to the manufacturer's instructions. Hex incorporation was accomplished by reaction of the growing chain with 18-dimethoxytrityl hexaethylene glycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. Biotinylation of oligonucleotide probes was achieved by incorporation of a biotin phosphoramidite. Oligonucleotides functionalised with alkaline phosphatase were prepared using the manufacturers proprietary method (Oswel). All oligonucleotides were HPLC purified using standard techniques.

Preparation of RNA

RNA was prepared using 1 pmol of probes 1 and 2, T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (25 units) and 2 µl rNTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37°C for 3 hours. DNA (probes 1 and 2) was removed from the assay mixture using RNase-free DNase (3 units DNase added per 20 µl assay mix, incubated at 37 °C for 10 minutes and 90 °C for 3 minutes).

Synthesis of RNA off hybridised oligonucleotide

Hybridisation was achieved in an assay mixture that contained 10 fmol of linear DNA template and 10 fmol of RNA complimentary to the 3' end of the linear template; Bst DNA polymerase (3' → 5' exo-, 4 units); 1 µl dNTP mix (2.5 mM of each dNTP: 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxythymidine 5'-triphosphate (dTTP), 2'-deoxyguanosine 5'-triphosphate (dGTP) and 2'-deoxycytidine 5'-triphosphate (dCTP)); T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (25 units) and 2 µl rNTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. Control reactions contained linear template without complimentary RNA. The mixture was incubated at 41°C for 3 hours.

Capture and detection of synthesised RNA

5 µl of assay sample was added to 145 µl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (specific for the RNA to be detected) and 6 pmol specific alkaline phosphatase oligonucleotide in streptavadin coated wells. Incubation (60 minutes at room temperature, shaking at 300 rpm) allowed the RNA to be immobilised on the wells via the biotinylated capture probe and annealed to the detection probe. Unbound material was removed from the wells by washing four times with TBS/0.1% Tween-20, then once with alkaline phosphatase substrate buffer (Boehringer Mannheim). Finally, alkaline phosphatase substrate buffer containing 4-nitrophenyl phosphate (5mg/ml) was added to each well. The plate was incubated at 37°C in a Labsystems EIA plate reader and readings were taken at 405 nm every 2 minutes. Results are presented in Figure 8.

List of oligonucleotides

H = Hex linker

Probe 1

5'TGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTCTCCCTATAGTGA
GTCGTATTAATTTC 3'

Probe 2

5'GAAATTAATACGACTCACTATA 3'

Sequence of transcribed RNA1

5'GGGAGACACAUCGGGUGAUAUCCAGAACGGAGACAAGGAGGCA 3'

Probe 3 (No Hex)

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGT
CTCCC 3' phosphate

Probe 4 (Hex between bases 12/13 from the 3' end);

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCTTGTCTCCGTTCTGGATATCACCCHGATGTGT
CTCCC 3' phosphate

Probe 5 (Hex between bases 24/25 from the 3' end);

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCTTGTCTCCGTTCHTGGATATCACCCGATGTGT
CTCCC 3' phosphate

Probe 6 (Hex between bases 36/37 from the 3' end);

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCHTTGTCTCCGTTCTGGATATCACCCGATGTGT
CTCCC 3' phosphate

Probe 7 (Hex in all 3 positions);

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCHTTGTCTCCGTTCHTGGATATCACCCHGATGT
GTCTCCC 3' phosphate

Capture Probe

5'TCTGCTGCCTGCTTGTCTGCGTTCT 3' (5' biotinylated)

Detection probe

5'GGATATCACCCG3' (3' alkaline phosphatase labelled)

Example 1a

This example demonstrated the use of Hexaethylene glycol (Hex) linkers as blockers of RNA polymerase single strand read through and other non-specific side reactions caused by nucleic acid polymerases. Hex linkers were incorporated between bases 2/3, 5/6, 7/8, 9/10 11/12 19/20 31/32 upstream from the –5 sequence which precedes the T7 promoter. In addition, Hex linkers were located between bases 6/7, 19/20 and 31/32 in the same template.

Preparation of oligonucleotides

All oligonucleotide probes were synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser according to the manufacturer's instructions. Hex incorporation was accomplished by reaction of the growing chain with 18-dimethoxytrityl hexaethylene glycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. Biotinylation of oligonucleotide probes was achieved by incorporation of a biotin phosphoramidite. Oligonucleotides functionalised with alkaline phosphatase were prepared using the manufacturers proprietary method (Oswel). All oligonucleotides were HPLC purified using standard techniques.

Preparation of RNA

RNA was prepared using 1 pmol of probes 1 and 2, T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (25 units) and 2 µl rNTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37°C for 3 hours. DNA (probes 1 and 2) was removed from the assay mixture using RNase-free DNase (3 units DNase added per 20 µl assay mix, incubated at 37 °C for 10 minutes and 90 °C for 3 minutes).

Synthesis of RNA off hybridised oligonucleotide

Hybridisation was achieved in an assay mixture that contained 10 fmol of linear DNA template and 10 fmol of RNA complimentary to the 3' end of the linear template; Bst DNA polymerase (3' → 5' exo-, 4 units); 1 µl dNTP mix (2.5 mM of each dNTP: 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxythymidine 5'-triphosphate (dTTP), 2'-deoxyguanosine 5'-triphosphate (dGTP) and 2'-deoxycytidine 5'-triphosphate (dCTP)); T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (25 units) and 2 µl rNTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. Control reactions contained linear template without complimentary RNA. The mixture was incubated at 41°C for 3 hours.

Capture and detection of synthesised RNA

5 µl of assay sample was added to 145 µl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (specific for the RNA to be detected) and 6 pmol specific alkaline phosphatase oligonucleotide in streptavadin coated wells. Incubation (60 minutes at room temperature, shaking at 300 rpm) allowed the RNA to be immobilised on the wells via the biotinylated capture probe and annealed to the detection probe. Unbound material was removed from the wells by washing four times with TBS/0.1% Tween-20, then once with alkaline phosphatase substrate buffer (Boehringer Mannheim). Finally, alkaline phosphatase substrate buffer containing 4-nitrophenyl phosphate (5mg/ml) was added to each well. The plate was incubated at 37°C in a Labsystems EIA plate reader and readings were taken at 405 nm every 2 minutes. Results are presented in Figure 9.

List of oligonucleotides

H = Hex linker

Probe 1

5'TGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTCTCCCTATAGTGA GTCGTATTAATTTC 3'

Probe 2

5'GAAATTAATACGACTCACTATA3'

Sequence of transcribed RNA1

5'GGGAGACACAUCGGGUGAUAUCCAGAACGGAGACAAGGAGGCA 3'

Probe 3 No Hex

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA GTCGTATTAATTTCTGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTC TCCC3'

Probe 4 Hex located between bases 31/32 from the -5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA GTCGTATTAATTTCTGCCTCCTTGTCTCCGTTCTGGATATCACCCHGATGTGT CTCCC3'

Probe 5 Hex located between bases 19/20 from the -5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA GTCGTATTAATTTCTGCCTCCTTGTCTCCGTTCHTGGATATCACCCGATGTGT CTCCC3'

Probe 6 Hex located between bases 11/12 from the -5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCTTGTHCTCCGTTCTGGATATCACCCGATGTGT
CTCCC3'

Probe 7 Hex located between bases 9/10 from the -5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCTTHGTCTCCGTTCTGGATATCACCCGATGTGT
CTCCC3'

Probe 8 Hex located between bases 7/8 from the -5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCHTTGTCTCCGTTCTGGATATCACCCGATGTGT
CTCCC3'

Probe 9 Hex located between bases 5/6 from the -5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTHCCTTGTCTCCGTTCTGGATATCACCCGATGTGT
CTCCC3'

Probe 10 Hex located between bases 2/3 from the -5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGHCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGT
CTCCC3'

Probe 11 Hex located between bases 6/7, 19/20 and 31/32

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCHTTGTCTCCGTTCHTGGATATCACCCHGATGT
GTCTCCC3'

Capture Probe

5'TCTGCTGCCTGCTTGTCTGCGTTCT 3' (5' biotinylated)

Detection probe

5'GGATATCACCCG3' (3' alkaline phosphatase labelled)

Example 2

This example demonstrated the use of Hex linkers in the stepped amplification of RNA. Two DNA templates were used. The first DNA template annealed RNA1, followed by extension by DNA polymerase and transcription of RNA2. The second template annealed RNA2 followed by extension by DNA polymerase and transcription of RNA1. Reactions were performed using two DNA templates without Hex linkers and with two DNA templates each containing three Hex linkers located between bases 12/13, 24/25 and 36/37 from the 3' end of the template.

Preparation of oligonucleotides

All oligonucleotide probes were synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser according to the manufacturer's instructions. Hex incorporation was accomplished by reaction of the growing chain with 18-dimethoxytrityl hexaethylene glycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. Biotinylation of oligonucleotide probes was achieved by incorporation of a biotin phosphoramidite. Oligonucleotides functionalised with alkaline phosphatase were prepared using the manufacturers proprietary method (Oswel). All oligonucleotides were HPLC purified using standard techniques.

Preparation of RNA

RNA1 was prepared using 1 pmol of probes 1 and 2, T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (25 units) and 2 µl rNTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37°C for 3 hours. DNA (probes 1 and 2) was removed from the assay mixture using RNase-free DNase (3 units DNase added per 20 µl assay mix, incubated at 37°C for 10 minutes and 90 °C for 3 minutes).

Synthesis of RNA off hybridised oligonucleotide

The reaction mixture contained 1 fmol of probe 3 (no Hexs) or probe 4 (three Hexs) and 1 fmol of RNA transcribed using probes 1 and 2. This RNA was complimentary to the 3' end of probes 3 and 4; Bst DNA polymerase (3' → 5' exo-minus, 4 units); 1 µl dNTP mix (2.5 mM of each dNTP: 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxythymidine 5'-triphosphate (dTTP), 2'-deoxyguanosine 5'-triphosphate (dGTP) and 2'-deoxycytidine 5'-triphosphate (dCTP)); T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (25 units) and 2 µl rNTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 41°C for 1 or 2 hours. At these intervals 100 fmol of probe 5 (no Hexs) or probe 6 (three Hexs) was added to the reactions containing probe 3 or probe 4, respectively. The mixture was reincubated at 41°C for a total reaction

time of 3 hours. Control reactions contained the same probes used in the full reactions but without complimentary RNA.

Capture and detection of synthesised RNA

5 µl of assay sample was added to 145 µl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (specific for the RNA to be detected) and 6 pmol specific alkaline phosphatase oligonucleotide in streptavadin coated wells. Incubation (60 minutes at room temperature, shaking at 300 rpm) allowed the RNA to be immobilised on the wells via the biotinylated capture probe and annealed to the detection probe. Unbound material was removed from the wells by washing four times with TBS/0.1% Tween-20, then once with alkaline phosphatase substrate buffer (Boehringer Mannheim). Finally, alkaline phosphatase substrate buffer containing 4-nitrophenyl phosphate (5mg/ml) was added to each well. The plate was incubated at 37°C in a Labsystems EIA plate reader and readings were taken at 405 nm every 2 minutes. Results are presented in Figure 10.

List of oligonucleotides

H = Hex linker

Probe 1

5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAATCGTCAGTCCCTATAGT
GAGTCGTATTAATTTC 3'

Probe 2

5'GAAATTAATACGACTCACTATA 3'

Sequence of transcribed RNA1

5'GGGACUGACGAUUCGGGUGAUAUCCAGAACGCAGACAAGCAGGCA 3'

Probe 3 (No Hexs)

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTGCTTGTCTGCGTTCTGGATATCACCCGAATCGTC
AGTCCC 3' phosphate

Probe 4 (Hexs between bases 14/15, 26/27 and 38/39 from the 3' end)

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTGC**H**TTGTCTGCGTTC**H**TGGATATCACCC**H**GAAT
CGTCAGTCCC 3' phosphate

Probe 5

5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAATCGTCAGTCCCTATAGT
GAGTCGTATTAATTTCTCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCT
CTCTTCC 3' phosphate

Probe 6 (Hexs between bases 14/15, 26/27 and 38/39 from the 3' end)

5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAATCGTCAGTCCCTATAGT
GAGTCGTATTAATTTCTCGTCT**H**TCCGGTCTCTCC**H**TCTCAAGCCTCA**H**GCG
TTCTCTCTTCC 3' phosphate

Capture Probe

5'TCTGCTGCCTGCTTGTCTGCGTTCT3' (5' biotinylated)

Detection probe

5'GGATATCACCCG3' (3' alkaline phosphatase labelled)

Example 3

This example demonstrated that a Hexaethylene glycol (Hex) linker located 9 bases upstream of the –5 sequence caused a reduction in background independent of the type of DNA polymerase used. Using either Bst, Klenow or Phi29 DNA polymerase for extension, a Hex linker lower the background using three different DNA templates.

Preparation of oligonucleotides

All oligonucleotide probes were synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser according to the manufacturer's instructions. Hex incorporation was accomplished by reaction of the growing chain with 18-dimethoxytrityl hexaethylene glycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. Biotinylation of oligonucleotide probes was achieved by incorporation of a biotin phosphoramidite. Oligonucleotides functionalised with alkaline phosphatase were prepared using the manufacturers proprietary method (Oswel). All oligonucleotides were HPLC purified using standard techniques.

Preparation of RNA

RNA was prepared using T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (25 units) and 2 µl rNTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37°C for 3 hours. DNA was removed from the assay mixture using RNase-free DNase (3 units DNase added per 20 µl assay mix, incubated at 37 °C for 10 minutes and 90 °C for 3 minutes). RNA1A was transcribed using 1 pmol probes 1A and 2A; RNA2A was prepared using 1 pmol probes 2A and 3A; RNA3A was prepared using 1 pmol probes 2 A and 4A.

Synthesis of RNA off hybridised oligonucleotide

Hybridisation was achieved in an assay mixture that contained 10 fmol of linear DNA template and 10 fmol of RNA complimentary to the 3' end of the linear template; Bst (3' → 5' exo-, 4 units), Klenow (3' → 5' exo-, 4 units) or Phi29 DNA polymerase (**3' → 5' exo-, 1 unit**); 1 µl dNTP mix (1.25 mM of each dNTP: 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxythymidine 5'-triphosphate (dTTP), 2'-deoxyguanosine 5'-triphosphate (dGTP) and 2'-deoxycytidine 5'-triphosphate (dCTP)); T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (25 units) and 2 µl rNTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. Control reactions contained linear template without complimentary RNA. The mixture was incubated at 41°C for 3 hours.

Capture and detection of synthesised RNA

5 µl of assay sample was added to 145 µl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (Capture probe 1 was used to detect the transcripts from Probes 9A and 10A; Capture probe 2 was used to detect the transcripts from Probes 5A, 6A, 7A and 8A) and 6 pmol alkaline phosphatase oligonucleotide (Detection Probe 1 was used to detect the transcripts from Probes 9A and 10A; Detection Probe 2 was used to detect the transcripts from Probes 5A, 6A, 7A and 8A) in streptavadin coated wells. Incubation (60 minutes at room temperature, shaking at 300 rpm) allowed the RNA to be immobilised on the wells via the biotinylated capture probe and annealed to the detection probe. Unbound material was removed from the wells by washing four times with TBS/0.1% Tween-20, then once with alkaline phosphatase substrate buffer (Boehringer Mannheim). Finally, alkaline phosphatase substrate buffer containing 4-nitrophenyl phosphate (5mg/ml) was added to each well. The plate was incubated at 37°C in a Labsystems EIA plate reader and readings were taken at 405 nm every 2 minutes. Results are presented in Figures 11a, 11b and 11c.

List of oligonucleotides

H = Hex linker

Probe 1A

5'TGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTCTCCCTATAGTGA
GTCGTATTAATTTC 3'

Probe 2A

GAAATTAATACGACTCACTATA

RNA1A

GGGAGACACAUCGGGUGAUAUCCAGAACGGAGACAAGGAGGCA

Probe 3A

5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT
GAGTCGTATTAATTTCTCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGT
CTCTTCC3'

RNA2A

5'GGAAGCGAGAACUCGGGUGAUAUCCAGAACGCAGACAAGCAGGCA 3'

Probe 4A

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCT
CGCTTCC3'

RNA3A

5'GGAAGAGACAACGCUGAGGCUUGAGAGGAGAGACCGGAAGACGA3'

Probe 5A

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTC
TCCC3'

Probe 6A Hex between bases 9/10 from the –5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGA
GTCGTATTAATTTCTGCCTCCTTHGTCTCCGTTCTGGATATCACCCGATGTGT
CTCCC3'

Probe 7A No Hex

5' TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCT
CGCTTCC 3'

Probe 8A Hex between bases 9/10 from the –5

5' TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTGCTTHGTCTGCGTTCTGGATATCACCCGAGTTC
TCGCTTCC 3'

Probe 9A No Hex

5' TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT
GAGTCGTATTAATTTCTCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGT
CTCTTCC 3'

Probe 10A Hex between bases 9/10 from the –5

5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT
GAGTCGTATTAATTTCTCGTCTTCCHGGTCTCTCCTCTCAAGCCTCAGCGTTG
TCTCTTCC 3'

Capture Probe 1

5' TCTGCTGCCTGCTTGTCTGCGTTCT 3' (5' biotinylated)

Detection probe 1
5' GGATATCACCCG3' (3' alkaline phosphatase)

Capture Probe 2

5' TCTGCTCGTCTTCCGGTCTCTCCTC 3' (5' biotinylated)

Detection probe 2

5' TCAAGCCTCAGC 3' (3' alkaline phosphatase)

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Claims

1. A method of detecting and amplifying a nucleic acid sequence of interest, the method comprising (a) contacting the sequence of interest with an oligonucleotide probe comprising sequences recognised by a nucleic acid polymerase and sequences complementary in part to the sequence of interest, so capable of hybridising thereto; (b) causing chain extension of at least one of the nucleic acids with a nucleic acid polymerase; (c) *De novo* synthesis of RNA from the functionalised nucleic acid polymerase sequence and; (d) further amplifying or detecting directly or indirectly the RNA thereto produced so as to indicate the presence of the sequence of interest; characterised in that the oligonucleotide probe comprises a linker/spacer moiety thereby preventing non-specific single strand nucleic acid readthrough by nucleic acid polymerases.

Figure 1

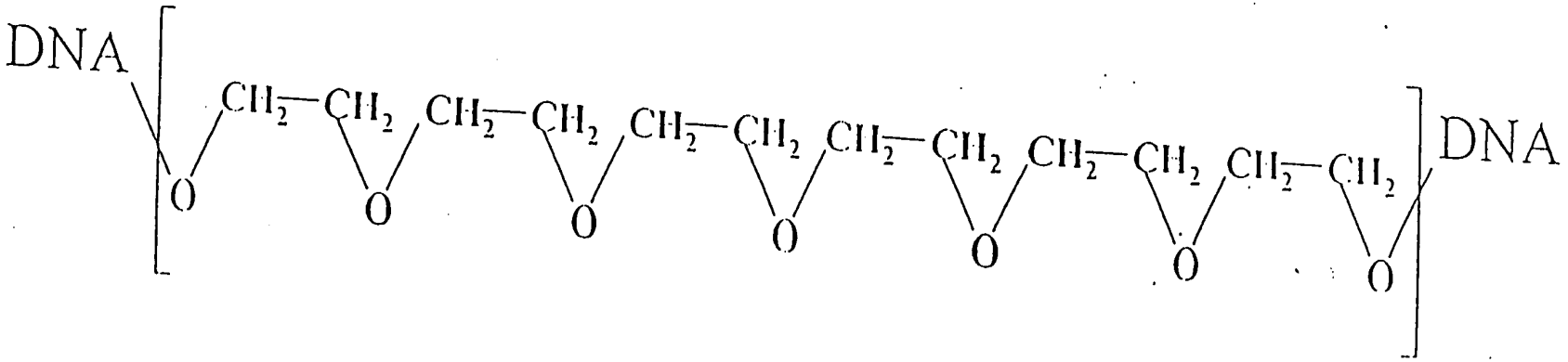


Figure 2

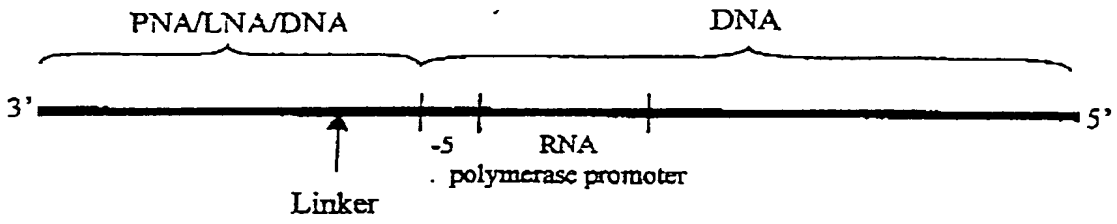


Figure 3

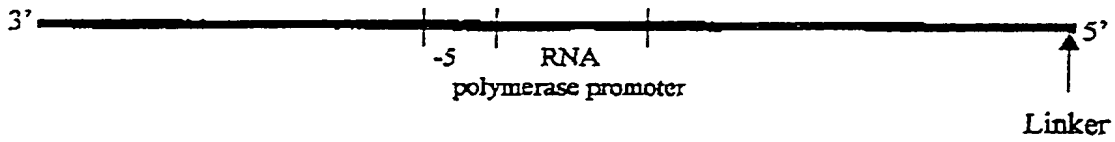


Figure 4

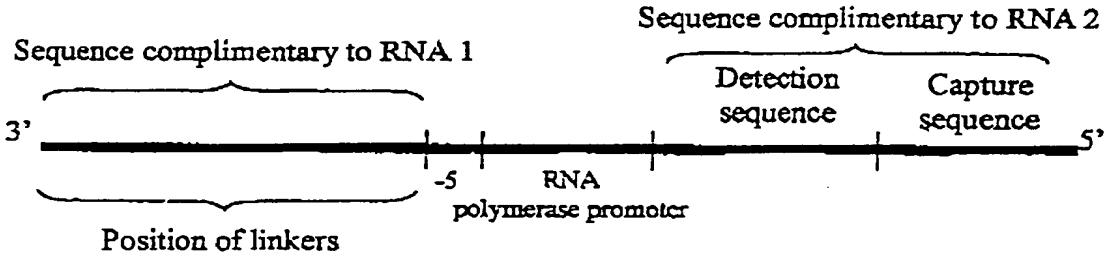


Figure 5

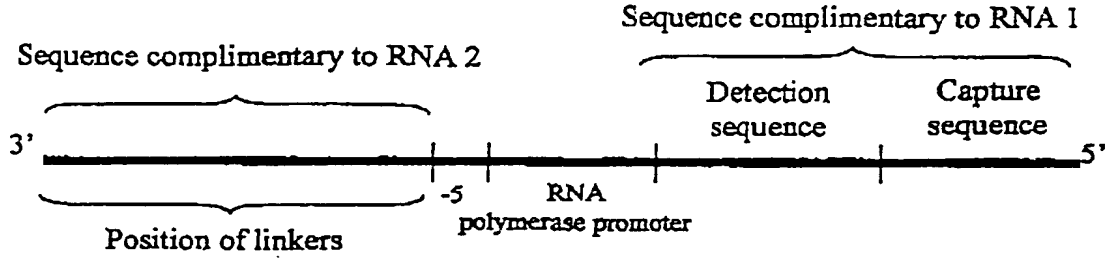


Figure 6

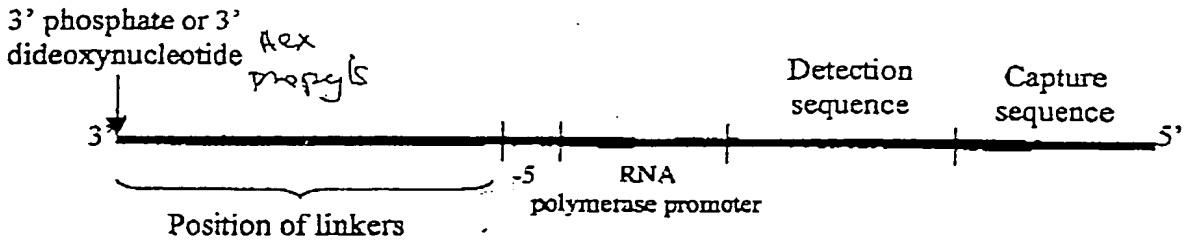


Figure 7

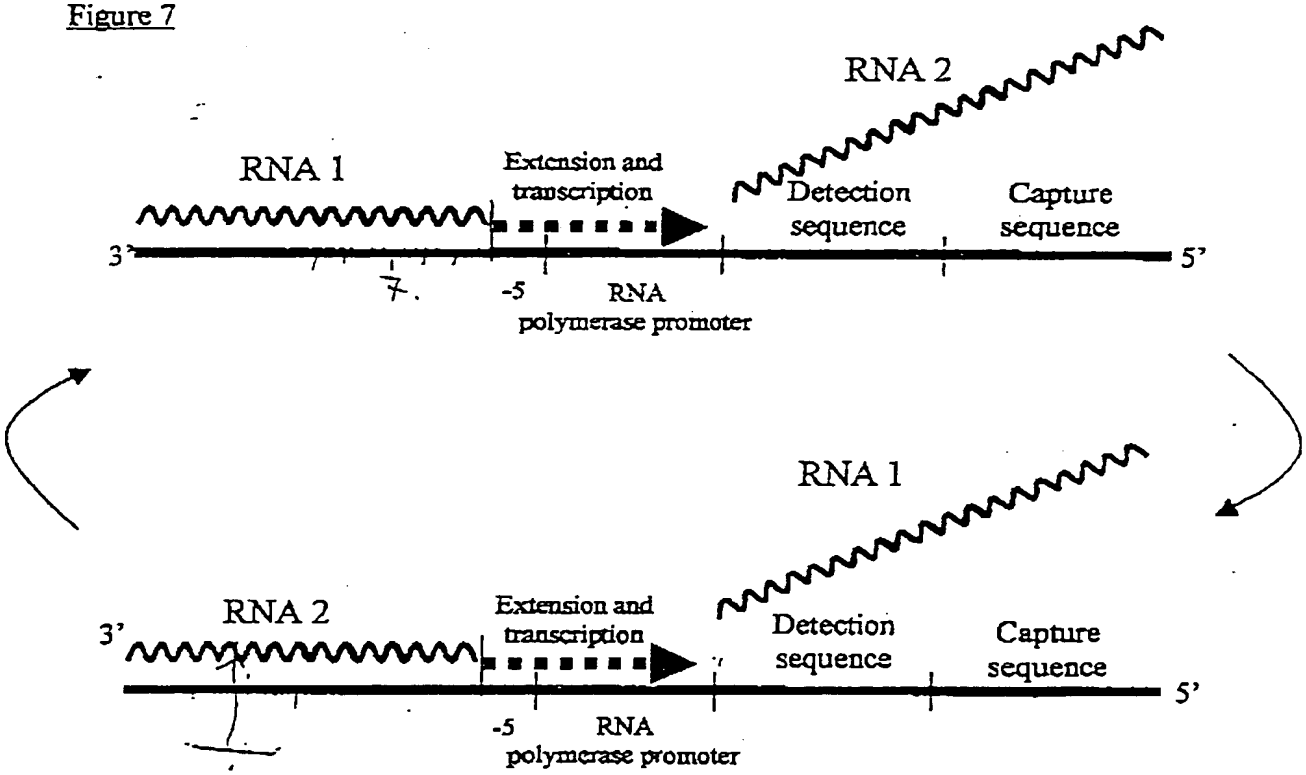
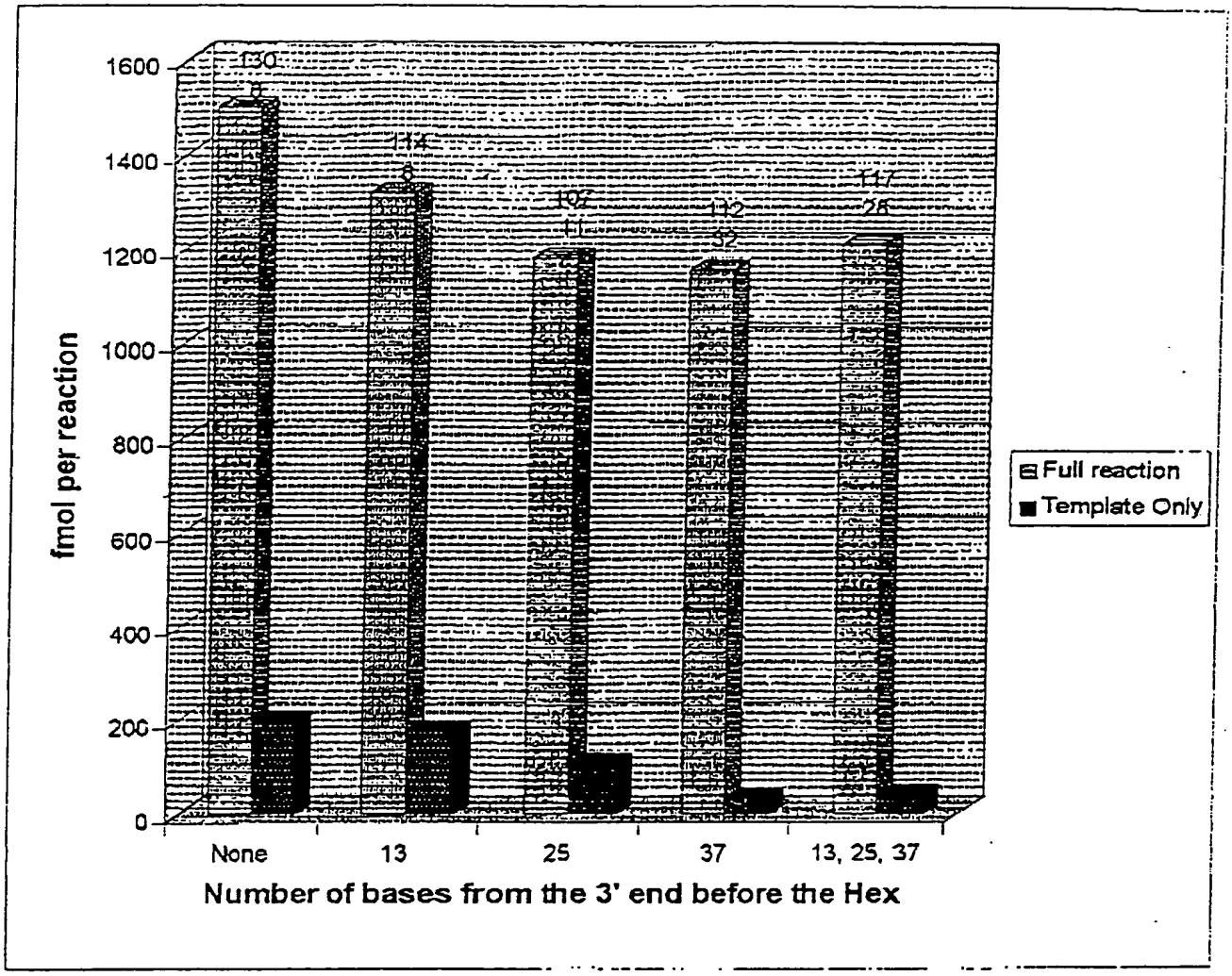


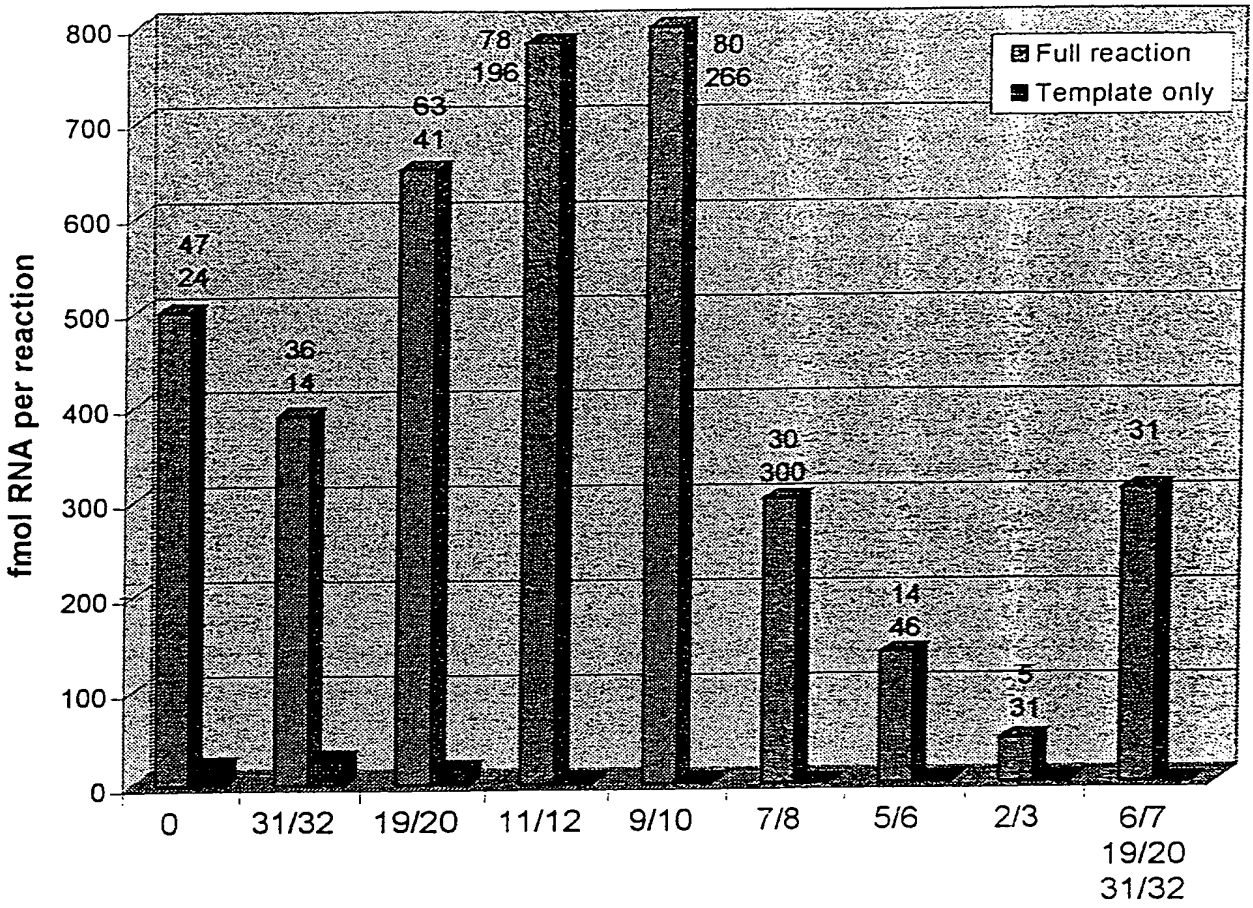
Figure 8 Effect of Hex linkers on extension and transcription of RNA from linear DNA templates.



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

FIG 8

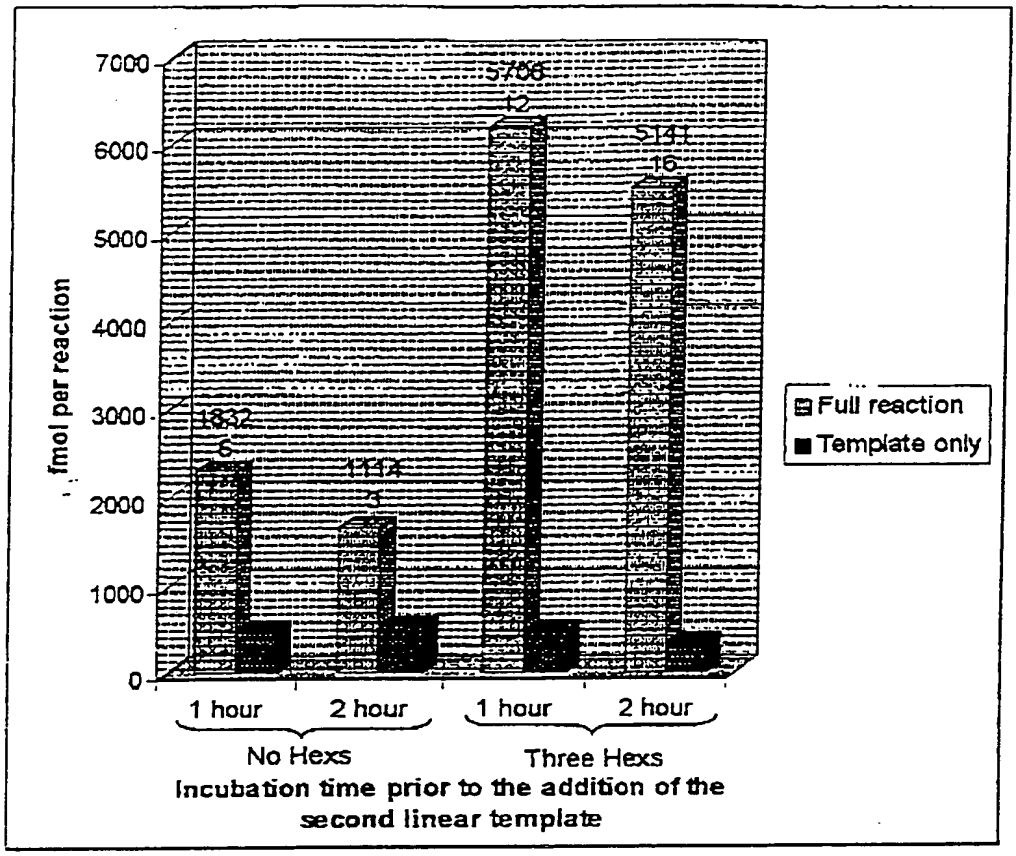
Figure 9: Extension and transcription of RNA from linear DNA templates containing Hex linkers.



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

FIG 9

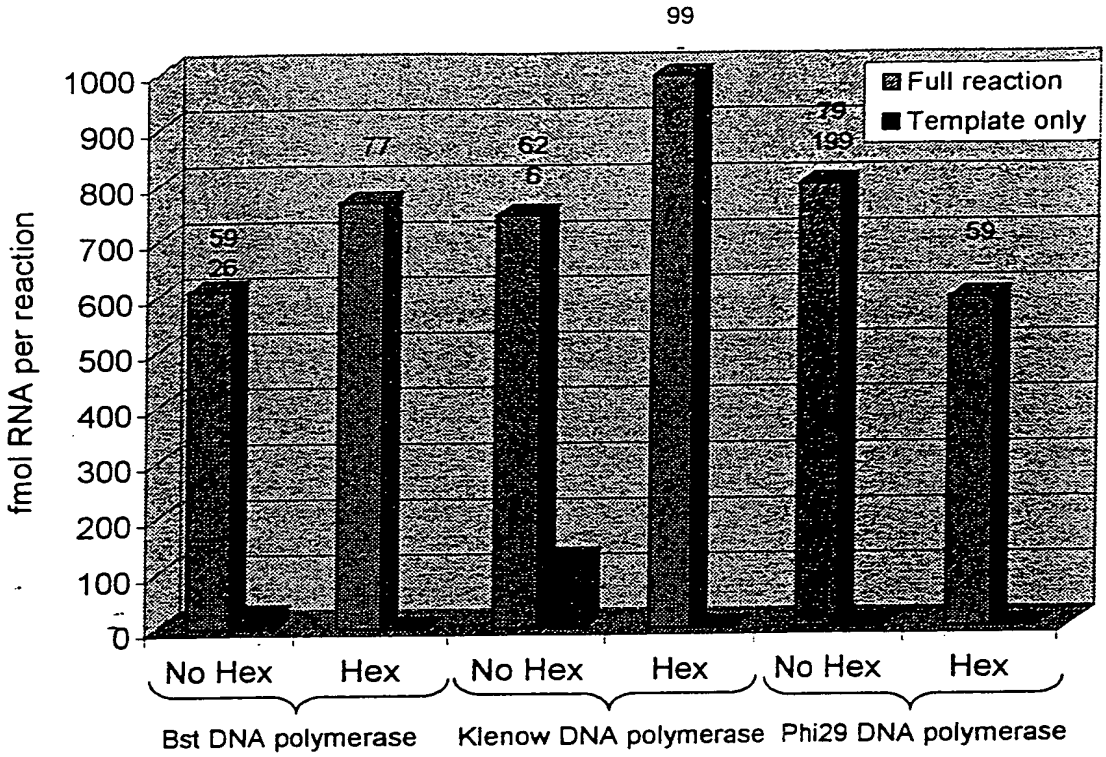
Figure 10 Stepped amplification using DNA templates with and without Hexs. The second DNA template was added after 1 or 2 hours.



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

FIG 10

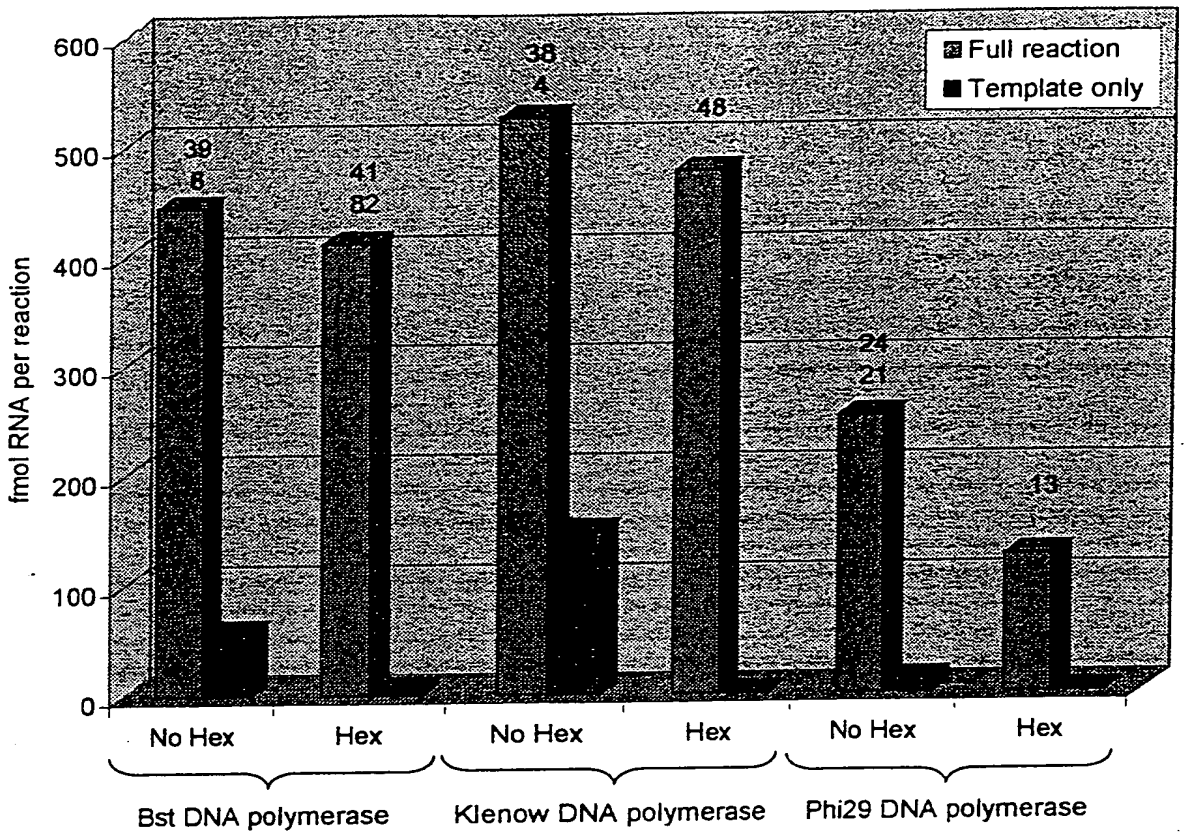
Figure 11a: Extension by three different DNA polymerases using RNA1A and Probes 5A (No Hex) or 5B (With Hex).



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

FIG 11a

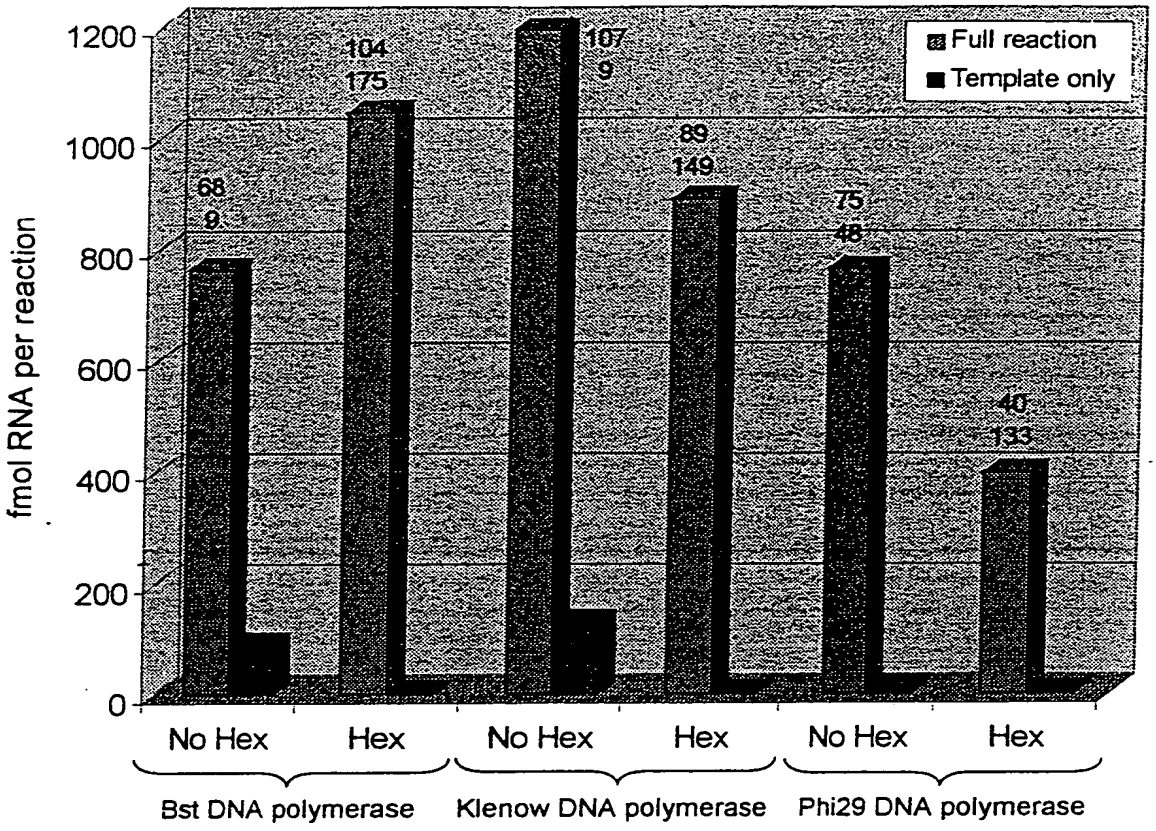
Fig 11b: Extension by three different DNA polymerases using RNA2A and Probes 7A (No Hex) or 8A (With Hex).



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

Fig 11b

Fig 11c: Extension by three different DNA polymerases using RNA3A and Probes 9A (No Hex) or 1[illegible] (With Hex).



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

FIG 11c

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